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Small Intestine Submucosa Effects on Long Term Viability of Transplanted Pancreatic Cells

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SMALL INTESTINE SUBMUCOSA EFFECTS ON LONG TERM VIABILITY OF TRANSPLANTED PANCREATIC CELLS.

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Pancreatic cell transplantation and the bioartificial pancreases can be used to treat diseases of the pancreas, such as pancreatitis and diabetes. However, there are a number of issues that limit the use of these techniques, including the limited quantity and variable quality of available cells and the patient's required immunosuppression. Therefore, this experiment sought to develop a method to encourage the proliferation of native pancreatic cells. Based on the promising results of a short term proof of concept study concerning pancreatic cell survivability when transplanted within an SIS packet, we developed a long term study to determine the viability of transplanted pancreatic cells. Twelve rats had SIS packets containing isolated pancreatic cells implanted in both their scrotum and abdomen. At 2, 3 and 4 month time intervals the rats were euthanized, the SIS packets were removed and prepared for microscopic examination using Maldonado's Staining Method. Upon examination of the SIS packet contents it was determined the abdominal packets exhibited an abundance of skeletal muscle cells while the scrotal packets contained mostly sebaceous glands. These finding were consistent for all time periods examined, with larger quantities of native cells being observed at the more distant endpoints. Though the methodology employed in this experiment failed to show the proliferation of transplanted pancreatic cells, the absence of such results may be

explained by extraneous factors. Therefore, while it is impossible within the confines of the current study to determine with absolute certainty if native cell proliferation affected pancreatic cell growth, an analysis of the various factors limiting cell proliferation implies that an additional study would benefit from shorter time intervals between SIS packet examinations.

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1.1. Overview

The biologic functions carried out by the pancreas are, without a doubt, essential to life. This can be demonstrated by the fact that some diabetics require insulin supplementation and those suffering from other diseases of the pancreas, such as pancreatitis, or who have undergone pancreatectomy require supplementation with exocrine enzymes. As would be expected, we are constantly searching for more long-term, patient oriented solutions to chronic health conditions. As such, there has been a gradual transition from daily insulin injections to insulin pumps which allow for easier and more closely controlled glucose levels. Work has also been done regarding various methods of pancreatic transplantation and with bioartificial pancreata, hoping to restore a degree of endocrine function (Kizilel, 2005). This research was designed to test the plausibility of using porcine small intestine submucosa as a medium on which pancreatic cells could proliferate and potentially be useful in transplantation or in a bioartificial pancreas.

1.2. Pancreatic Function

The pancreas is an organ composed of two distinct parenchymal tissue types which makes it a dual-function organ intimately involved in the production and secretion of both digestive enzymes and hormones. Digestive enzymes, such as trypsin,

chymotrypsin, pancreatic amylase and pancreatic lipase, are produced in the acini of the exocrine pancreas and secreted into the small intestine. These enzymes are instrumental in the breakdown of protein, starch, and fat. The endocrine pancreas is composed of the Islets of Langerhans which contain four main cell types: α cells which secrete glucagon, β cells which secrete insulin, δ cells which secrete somatostatin and gastrin, and PP cells which secrete pancreatic polypeptide (Figure 1). These hormones help control blood glucose levels, growth and the release of gastric acid (Silverthorn 2004).

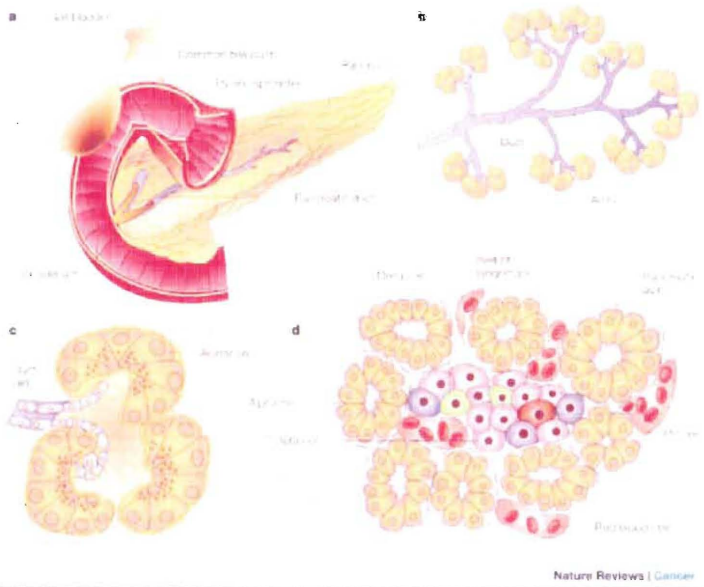

Figure 1: Anatomy of the pancreas (Bardeesy 2002).

Since pancreatic secretions are instrumental to many of the body's functions, it is easy to understand how crucial the pancreas is to leading a normal productive life and why so much time and energy has been invested in the quest for viable treatments of pancreatic dysfunctions. Pancreatic diseases can have far ranging effects on the affected individual so it is important to learn as much as possible about the causes and consequences of these diseases and continue the search for more patient friendly treatments.

1.3.1. Diseases of the Pancreas: Diabetes

Diabetes is a disease of the pancreas dealing in particular with a deficiency of insulin due to poor insulin production, action, or a combination of the two, and is characterized by high levels of blood glucose. Blood glucose levels are high because the effected hormone, insulin, is responsible for regulating the removal of glucose from the blood/entry of glucose from the blood into the cells. Diabetes can be further subdivided into specific categories, mainly types 1 and 2, although various other types of diabetes do exist. Type 1 or insulin dependent diabetes is caused by the autoimmune destruction of the insulin producing β cells in the patient's pancreas. Type 2 or insulin independent diabetes, which accounts for 90-95% of all cases of diabetes (American Diabetes Association, 2010), generally begins as insulin resistance and gradually progresses as the pancreas looses the ability to secrete insulin (Silverthorn, 2004). Type 2 diabetes is generally associated with older age, obesity, family history, ethnicity and physical inactivity (American Diabetes Association, 2010).

According to the American Diabetes Association, 20.8 million people, or 7% of the population, have diabetes while nearly 1/3 of them are unaware that they have the disease. The socioeconomic cost of diabetes totaled $174 million in 2007, $116 billion of which was medically related while the remaining $58 billion was lost due to days missed from work or poor work performance as a result of the disease. Diabetes is the 5th deadliest disease in the United States, having killed 224,092 people in 2002 (American Diabetes Association, 2010).

1.3.2 Diseases of the Pancreas: Standard Treatment for Diabetes

Type 1diabetes is most commonly treated with insulin therapy due to a type 1 diabetic's lack of insulin. Type 2 diabetics have a much wider range of treatment options and frequently combine any of a number of therapies, including insulin, to properly manage their condition. The one common denominator of all of these therapies is that their mechanisms of action revolve around the function of insulin. Thiazolidinediones increase the body's sensitivity to insulin, whereas the release of insulin is increased by sulfonylureas (Davis). Biguanides and acarbose decrease the need for insulin by affecting hepatic and gastrointestinal function (Davis). A number of other lifestyle changes, such as weight loss, can greatly increase the effectiveness of type 2 diabetes management (Powers). When treating diabetics one must also be aware of other potential complications of diabetes, such as nephropathy, retinopathy and cardiovascular disease. It is important to remember that while insulin and other medications are used to help manage diabetes, and much is known about possible complications of diabetes, there is, as of yet, no known cure for diabetes itself.

1.3.3. Diseases of the Pancreas: Pancreatitis

Pancreatitis is a disease in which the pancreas' own digestive enzymes become active within the organ itself, beginning the process of autodigestion and causing an immense amount of pain for the patient. There are two distinct types of pancreatitis, acute pancreatitis which is usually reversible and chronic pancreatitis which is generally

irreversible. Both classes of pancreatitis have seen an increased prominence over the last decade and represent a major financial burden on the healthcare system (Bell 2001, Fagenholz 2007, Ginsberg 2006, Uhl 1999).

Acute pancreatitis is usually caused by chronic alcohol intake or gallstones, which combined account for 90% of acute pancreatitis cases. Other minor causes of the disease include hyperlipidemia, hypercalcemia and adverse reactions to medications (Fagenholz 2007). In 1997 U.S. physicians diagnosed 263,136 people with acute pancreatitis accounting for a medical expenditure of $4.8 billion (Bell 2001). The rate of acute pancreatitis is highest in the United States when compared to the rest of the Western world (Fagenholz 2007).

Chronic pancreatitis accounts for an additional 122,000 outpatient visits and 56,000 hospitalizations a year with a cost of approximately $2.1 billion. Just as alcohol abuse can cause acute pancreatitis, prolonged use of alcohol can also cause chronic pancreatitis. Therefore, it is not uncommon for a patient to progress from isolated cases of acute pancreatitis to chronic pancreatitis if alcohol intake is not curtailed (Ginsberg 2006). Other causes of chronic pancreatitis include pancreatic cancer, hypercalcemia, renal failure, gallstones, pancreatic trauma and hyperlipidemia (Stevens).

1.3.4. Diseases of the Pancreas: Standard Treatment for Pancreatitis

The initial treatments for both chronic and acute pancreatitis are essentially the same. If gallstones, hypercalcemia or hyperlipidemia are the cause those etiologic factors are treated. Pain is controlled through medications and rarely via nerve blockade. As a

precautionary measure antibiotics may be ordered, as can supplemental pancreatic enzymes to promote pancreatic rest. The digestive system may also be rested, although sometimes enteral nutrition is preferred over parenteral nutrition. Aggressive fluid resuscitation and rehydration is also very important as is abstinence from alcohol (Carroll 2007, Ginsberg 2006). Surgery may sometimes be necessary but generally is ordered no sooner than 2 weeks after the onset of symptoms. (Uhl 1999, Carroll 2007, Khoury 2010, Stevens, Blondet 2007)

Surgical treatment options for pancreatitis include a variety of debridement procedures, partial pancreatic resections and total pancreatectomy. As of yet there is no ideal surgical procedure as the efficacy of most procedures at relieving symptoms is no greater than 85-90%, oftentimes resulting in the need for numerous surgeries before the patient experiences relief of symptoms. (Uhl 1999, Wani 2007). However, the need for surgical debridement is now universally accepted, with the preference being to preserve as much of the pancreas as possible through limited or partial resection (Uhl 1999). One potential and almost universal complication of pancreatic resection is surgical diabetes since the patient loses most, if not all, of their endocrine function following surgery, effectively substituting one pancreatic disease for another. It is therefore in our best interest to develop a means by which we can provide pancreatitis patients, diabetics and anyone else suffering from the wide range of pancreatic maladies a way to recover or replace some of their lost pancreatic function.

1.4. Bioartificial Pancreas

The desire to replace or augment failing pancreatic function has led the scientific community to explore numerous possible therapies with the hope of improving the quality of life for a patient suffering from decreased pancreatic function. One such intensely researched therapy is the bioartificial pancreas (BAP). The method of construction and theory behind the functioning of all BAP devices are essentially the same. A sufficient number of pancreatic islet cells are placed within the BAP which has a semipermeable membrane possessing pores of sufficient size to allow oxygen and other nutrients, as well as insulin to pass, while preventing the passage of the majority of immunocytes (Ikeda 2006, Iwata 2004, Kizilel 2005). This device can then be placed directly in line with vascular flow or in a well vascularized area of the body, such as the abdominal cavity, thereby nourishing the islets and allowing insulin to enter the blood stream. While BAPs have experienced a moderate amount of success using islets from xenogenic, as well as cadaveric donors, there are a number of obstacles that need to be overcome (Iwata 2004, Ikeda 2006).

One such obstacle is the construction of the BAP itself. Currently, there is no device that is effective enough, or safe enough to warrant its widespread use. Many BAPs suffer from poor diffusion ratios. This is because the large amount of pancreatic tissue the BAP houses has a very large oxygen requirement but BAPs generally have a very small surface area. This does not allow the proper exchange of nutrients, waste products and hormones with the blood, eventually causing the device to fail (Thorens 2007). Additionally, the materials with which current BAPs are constructed are rather fragile. It

is not uncommon for these devices to rupture, thereby spilling their contents out into the patient's body and initiating a huge immune response (Ikeda 2006, Iwata 2004). This possibility, coupled with the fact that it is impossible to keep every immunocyte from passing through the BAP's semipermeable membrane, necessitates an immunosuppressive drug regimen, accompanied by all its side effects, for patients (Ikeda 2006, Iwata2004, Kizilel 2005).

Another major obstacle preventing the widespread use of BAPs is a lack of sufficient islet cells to place within the BAP. Researchers have used porcine islets in a number of experiments to test the viability of BAPs but are generally afraid to suggest their widespread use in human patients. Although these islets would produce insulin that closely resembles human insulin and is proven to function in the human body there is speculation that porcine endogenous retroviruses could be transmitted to the patient (Ikeda 2006). While human islets from cadaveric donors have been successfully used there is severe shortage of available organs (Roche 2005, Kizelil 2005). This shortage is further exacerbated by the fact that two pancreases from brain dead donors are generally required to acquire enough viable islet cells to enable the patient to return to a euglycemic state. For that reason it is essential that we find a renewable source of cells, thereby allowing us to circumvent cell shortage problems (Roche 2005, Iwata 2004).

1.5 Islet Transplantation

Another of the many therapies currently under investigation is islet transplantation. Islet transplantation is preferred to pancreas transplantation because it is a

less invasive procedure. Islet cells make up approximately 1% of the total pancreatic mass. Therefore, when islet cells are transplanted, as opposed to the entire organ, the potential for an immune response and the risk associated with the procedure itself is reduced (Roche 2005, Meloche 2007). Just as the bioartificial pancreas faces a number of challenges so does islet transplantation. In fact, the issues facing both techniques are essentially the same and are shared with any sort of transplantation procedure. In islet transplantation the lack of a sufficient number of organs manifests itself as inadequate islet cell mass and inadequate islet cell potency due to the propensity to obtain these cells from deceased donors. Other issues include graft rejection and the need for a lifelong immunosuppressive drug regimen (Bretzel 2007). Even though islet transplantation must overcome a litany of issues it still remains an attractive and often preferred method of treatment for diabetic and pancreatitis patients alike.

Numerous studies demonstrate the benefits of islet transplantation when performed in conjunction with pancreatic resection (Robertson 2001b, Blondet 2007, Panaro 2004, Warnock 2005, Gruessner 2003). While relatively few such procedures have been performed, pancreatic resection with auto-islet transplantation has been successful in preventing surgical diabetes. In one case, surgical diabetes had not been documented 13 years post-operatively (Robertson 2001b), which as of 2007 had increased to 20 years (Blondet 2007). Such findings have led auto-islet transplantation to be billed as "the gold standard" treatment for the preservation of endocrine function after pancreatic resection (Panaro 2004). Similarly, one study determined that islet allotransplantation when compared to the best current medical therapy for diabetes produced similar results without the need for insulin injections (Warnock 2005). Due to

these successes and the fact that auto-islet transplantation does not require immunosuppressive drugs it is quickly becoming the pancreatic transplantation procedure of choice, being performed whenever resources allow (Gruessner 2003).

1.6 Auto-Islet Transplantation

While rare, approximately 400 auto-islet transplantation procedures have been performed worldwide as of 2005, the majority of these procedures follow total or near total pancreatic resection for chronic pancreatitis instead of the more tradition pancreatic transplant (Roche 2005, Gruessner 2003, Farney 1998, Berney 2004). As stated above, this procedure has numerous advantages over other transplantation procedures such as decreased chance of graft rejection, no need for immunosuppressive drugs and fewer surgical risks. However, the most striking aspect of this procedure is that it makes use of the patient's own islet cells, eliminating the need for deceased, living or xenogenic donors.

As is the case with other islet transplantation procedures, success is dependent upon transplanting a sufficient number of cells to achieve insulin independence (Morrison 2002, Blondet 2007). In this respect autotransplantation once again has a number of factors working in its favor. Autoislet transplantation has successfully restored normal glucose tolerance in chronic pancreatitis patients using far fewer cells than successful allotransplant procedures (Illouz 2007). While allotransplantation procedures frequently require 13,000 islet equivalents (IEQ) or more per kilogram of body weight (Shapiro 2006) autotransplantation require less than 3000 IEQ/kg (White 1998). This

discrepancy in the number of islets required can be explained by the fact that immunosuppressive drugs are not present and are therefore not toxic to the islets (Meloche 2007). Additionally, the islets came from a living, and presumably healthier donor, thereby reducing both warm and cold ischemic times, and damage to the islets themselves (Robertson 2001a). This finding is further bolstered by research indicating that living donors are the best source of insulin-producing islets for transplantation procedures (Jung 2007).

Due to the success of islet transplantation it is necessary to work toward improving islet cell yield so that more patients can benefit from islet cell transplantation. One barrier to improving the number of islet cells isolated is the prevailing medical theory which dictates delaying surgical resection of the pancreas as long as possible to preserve endocrine and exocrine function (Robertson 2001b). However, this delay in resection results in a highly necrotic pancreas by the time surgery is considered, thereby eliminating the possibility of an autotransplantation due to a scarcity of viable pancreatic cells (Morrison 2002). Therefore, it is necessary to change the prevailing medical doctrine concerning chronic pancreatitis and surgery in favor of earlier surgeries (Blondet 2007), while developing a method by which we could maximize the number of viable islet cells available from living donors.

1.7 Small Intestine Submucosa

While all animals possess small intestine and therefore small intestine submucosa (SIS), it is important to note that not all SIS is structurally similar. When SIS xenografts

of feline, porcine, equine, ovine, caprine, bovine and human origin were tested for suitability as a femoral graft, only two proved successful, feline and porcine. The other SIS materials failed due to graft occlusion, poor suture retention or a high degree of handling difficulty (Lantz 1993). As a result of this study and the ready supply of porcine SIS the majority of research concerning the properties of SIS, as well is effectiveness in various scenarios, has centered on porcine SIS.

Porcine small intestine submucosa possesses many unique qualities that make it especially well suited to perform as a bioscaffold for tissue regeneration. As a collagen based acellular biological material, SIS poses little to no threat of infection, rejection or inflammation to any host organism (Poulose 2005, Lantz 1993 and Kim 2007). It also possesses the unique ability to regenerate native tissue, including the required vascular structures for nourishment of those tissues, while at the same time removing itself from the host organism (Poulose 2005). The ability of SIS to regenerate tissue can be explained by the fact that it contains numerous growth factors, namely FGF-2 and TGF-β1, which play significant roles in tissue remodeling and wound healing (Kim 2007 and Voytik-Harbin 1997). FGF-2 is responsible for stimulating the proliferation of fibroblasts, vascular endothelial cells, smooth muscle cells, chondrocytes and osteoblasts. Similarly, TGF-β1 stimulates the migration of monocytes, lymphocytes, neutrophils and fibroblasts. In addition, TGF-β1 has multiple effects on the extracellular matrix, controlling both its manufacture and degradation by regulating the transcription of collagen, fibronectin, glycoaminoglycans and various proteases (Voytik-Harbin 1997). Just as the growth factors contained in SIS make it well suited to help regenerate tissue so do the constituent glycosaminoglycans. Heparin and heparin sulfate are required by

growth factor FGF-2 to bind to its receptors, while hyaluronic acid promote scarless wound repair by binding TGF-β1. Dermatan sulfate possesses anticoagulant properties which help to explain the thromboresisant properties observed when used as a vascular graft (Hodde 1996).

SIS has successfully been used as graft material for numerous tissue types in a number of different animal models. These tissue types include but are not limited to large and small diameter vascular grafts (Lantz 1993 and Badylak 1989), urinary bladder wall (Kropp 2000, 2004), the biliary tract (Rosen 2002), Achille's tendon (Badylak 1995), the ureter (Jaffe 2001), uterine horn (Taveau2004) and sciatic nerve (Smith 2004). Of particular relevance to this study is previous research in which rats were the model organism. Such research includes detrusor muscle regeneration (Vaught 1996), bladder wall regeneration (Kropp 2004) and SIS as a microvascular graft (Prevel 1994). In each of these cases the experimenters reported the successful use of porcine SIS as a graft material. Success was defined as detrusor muscle that was contractile and functionally innervated, a bladder with near normal function or microvasculature that remained patent for a number of hours. It is important to note that at no time was there any evidence of graft rejection or infection when porcine SIS was used in a rat model. Additionally, it has been proven that SIS has the ability to improve islet function and survivability in vitro. Rat islets cultured on SIS and allowed to incubate for 7-14 days exhibited survivability and functionality as evidenced by insulin secretion (Xiaohui 2006). Interestingly, human islets also exhibited the above characteristic when cultured on sheet-formed SIS proprietarily modified by Cook Biotech (Woods 2004). This study was developed in an

attempt to build upon the successful use of SIS in general and, more specifically, on the use of SIS in rat models and in vitro use of SIS with pancreatic islet cells.

Our laboratory has successfully used SIS in a number of applications, including as an aortic graft, ureteral segment replacement, uterine horn regeneration and as a nerve conduit. It was determined that SIS serves as a feasible aortic graft in a swine model. When the grafts were microscopically examined 28 days post surgery they had undergone reendothelialization with neovascularization. No indication of rejection or infection was observed, the latter of which is attributed to the preoperative cold-sterilization procedure followed (Marshall 2000). When SIS was used to replace a segment of the ureter in New Zealand white rabbits, urothelial cells lined the lumen, and a small amount of smooth muscle fibers and blood vessels were present 11 days following the surgery. Thirty five days following the surgery the same tissue types were more prevalent and well organized further demonstrating SIS's ability to epithelialize when placed within the ureter (Jaffe 2001). Once again illustrating that SIS proved capable of regenerating tissue, this time uterine horn tissue in New Zealand white rabbits, researchers used staining and electron microscopy techniques to determine that epithelium, muscular and serosal layers were present at the site of the SIS graft in the uterine horn. In order to prove that the graft functioned properly the rabbits were mated, with half, generally those with a shorter graft, becoming pregnant. This once again demonstrated that epithelium and other tubular morphology could be regenerated using SIS (Taveau 2004). The nerve regenerative ability of SIS was tested on the sciatic nerve of rats. After a 90 day period in which regeneration had the opportunity to occur myelination and tubulin activity in the proximal

portion of the graft was noted. This study identified SIS as a possible material with which to aid nervous tissue regeneration (Smith 2004).

1.8. Animals

Since the objective of this study was to determine the ability of SIS to maintain the viability of transplanted pancreatic cells over the long term it was important to test the SIS/pancreatic cell combination within a living organism. The rat model was chosen for this project because it is the smallest mammalian species possessing easily accessible, well vascularized sites for the implantation of SIS packets. After consultation with the project veterinarian two implantation sites were chosen: the abdomen and scrotum. The use of two standard implantation sites allowed for a statistical comparison of the respective sites' ability to maintain the viability of the SIS packet. A certain degree of variability was eliminated by placing an SIS packet at each location in every recipient rat.

1.9. Proof of Concept

Building upon the success experienced by researchers both within and outside of our laboratory, we decided to test the ability of SIS to support transplanted pancreatic cells. The initial study, entitled *Will Small Intestine Submucosa Support Pancreas Cell Transplantation*, conducted by Guy David Prosper at the Philadelphia College of Osteopathic Medicine, determined that SIS is a viable bioscaffold for pancreatic cells and is capable of supporting those cells over the short term in an in vivo model (unpublished

laboratory data). In that study SIS packets containing isolated pancreatic cells were placed in the abdomen or scrotum of 12 recipient Sprague Dawley rats that then bore the implant for 4 weeks. At 4 weeks post surgery the SIS packets were removed and examined, determining that the SIS was still viable and beginning to remodel into native tissue as neovascularization and connective tissue ingrowth was noted. Additionally, a small number of pancreatic cells were observed in the tissue samples. This previous study provided the theory upon which the current study was based as well as the basis from which the methods were developed. Some results from the proof of concept study are included within this text with the consent of Guy David Prosper.

1.10 The Present Study

While at the end of the 1 month proof of concept study cell proliferation and neovascularization was observed, in other studies it was noted that complete tissue remodeling may take months and possibly years to be complete. For that reason, a long term study was designed to determine the ability of SIS to maintain the viability of transplanted pancreatic cells over the long term. In this study we chose to examine the SIS packets and their contents 2, 3 and 4 months after implantation. When this data was combined with that of the one month study, we were able to observe the remodeling process in 1 month intervals for 4 months, providing us with an excellent picture of the ability of SIS to maintain pancreatic cells as it ages, remodels and is eventually replaced by native tissue.

MATERIALS AND METHODS

2.1 Objectives

This research is designed as a follow up study to an Institutional Animal Care and Use Committee approved proof of concept study previously conducted in our laboratory which showed positive results out to 1 month post surgery (unpublished laboratory data). The present study seeks to extend the length of time the SIS packets are left implanted in the recipient animal to determine the long term survivability of transplanted pancreatic cells at 2, 3 and 4 month intervals, and the possible advantage of one implantation site, abdomen or scrotum, over the other. Extending the length of the experiment, and therefore the growth period for pancreatic cells and SIS remodeling, agrees with the protocols and findings of other researchers (including previous experiments conducted in this lab in other tissue types) where a growth period of 3 to 6 months, in some instances a year or more, was allowed to elapse before checking for graft rejection, other immune responses or the progress of the remodeling process (Robertson 2001b, Soon-Shiong 1987 & Gruessner 2006).

The specific aims of this research were: (1) to create a cellular packet using SIS; (2) harvest viable rat pancreatic cells; (3) place packets of rat pancreatic cells into the abdomen or scrotum of recipient rats (4) determine if pancreatic cells maintain their viability and proliferate over the course of four months.

2.2 Hypothesis

We had reason to anticipate a successful study due to the promising results of a short term proof of concept study concerning pancreatic cell survivability when transplanted within an SIS packet. We therefore expected to see parenchymal cell proliferation resulting in partial remodeling of the transplanted SIS into viable, pancreatic tissue accompanied by neovascularization of the transplant site.

2.3. SIS Packet Preparation

Porcine small intestine was obtained from a USDA approved vendor in the fresh state. The jejunum was identified, separated from the rest of the small intestine, cleaned and stored in a 10% gentamicin, physiological saline solution.

To begin the construction of the SIS packet a small portion of jejunum was transected and cut longitudinally in order to form a sheet. The mucosal surface was denuded while the serosa and muscularis were peeled away using forceps. This isolated SIS sheet was then cut into 1x2 cm strips. A tapered needle with 6.0 Vicryl suture was pushed through one of the top corners of the sheet and then through the bottom corner on the ipsilateral side, leaving a loop of suture material on the top, serosal, surface of the SIS sheet. This process was repeated on the opposite side of the sheet (Figure 2-A). These loops of suture material were then placed around a section of 5mm diameter glass stirring rod which was held securely in placed by a ring stand and clamp. The suture was used to position the SIS on the glass rod, stretching it out and bringing the ends into apposition

while placing the serosal side against the glass rod (Figure 2-B). At this point the suture material was tied off at each end, while leaving a length of suture to enable further manipulation of the SIS and facilitate the implantation process (Figure 2-C). While the SIS was held taut by putting tension on the remaining suture material, sutures were placed along the length of the SIS packet, creating a continuous tube of SIS (Figure 2-D&E). Another length of suture was then passed along the length of SIS tube to enable it to be manipulated while sutures were being placed in the two remaining corners. When these sutures were secure the additional length of suture material was removed (Figure 2-F). As previously, the corner sutures were retained at a sufficient length to enable manipulation of the SIS tube as it was modeled into an SIS packet. The SIS tube was then positioned so that an open end was facing outward (Figure 2-G). The open end was sutured closed and the SIS was repositioned so that the other open end faced outward (Figure 2-H). The remaining open end was sutured 1/3 closed, leaving an opening for the injection of the isolated pancreatic cells. Half of the SIS packets were retained with 2 lengths of suture material with needles still attached in order to secure those packets in the abdominal cavity. The other packets had all excess suture material removed as those packets were intended for implantation in the scrotum and did not need to be sutured in place. Throughout this process it was necessary to keep the SIS, suture and glass stirring rod moist using saline solution to avoid tearing and desiccation of the SIS. All packets were maintained in a 10% gentamicin/saline solution and refrigerated until the implantation procedure was performed.

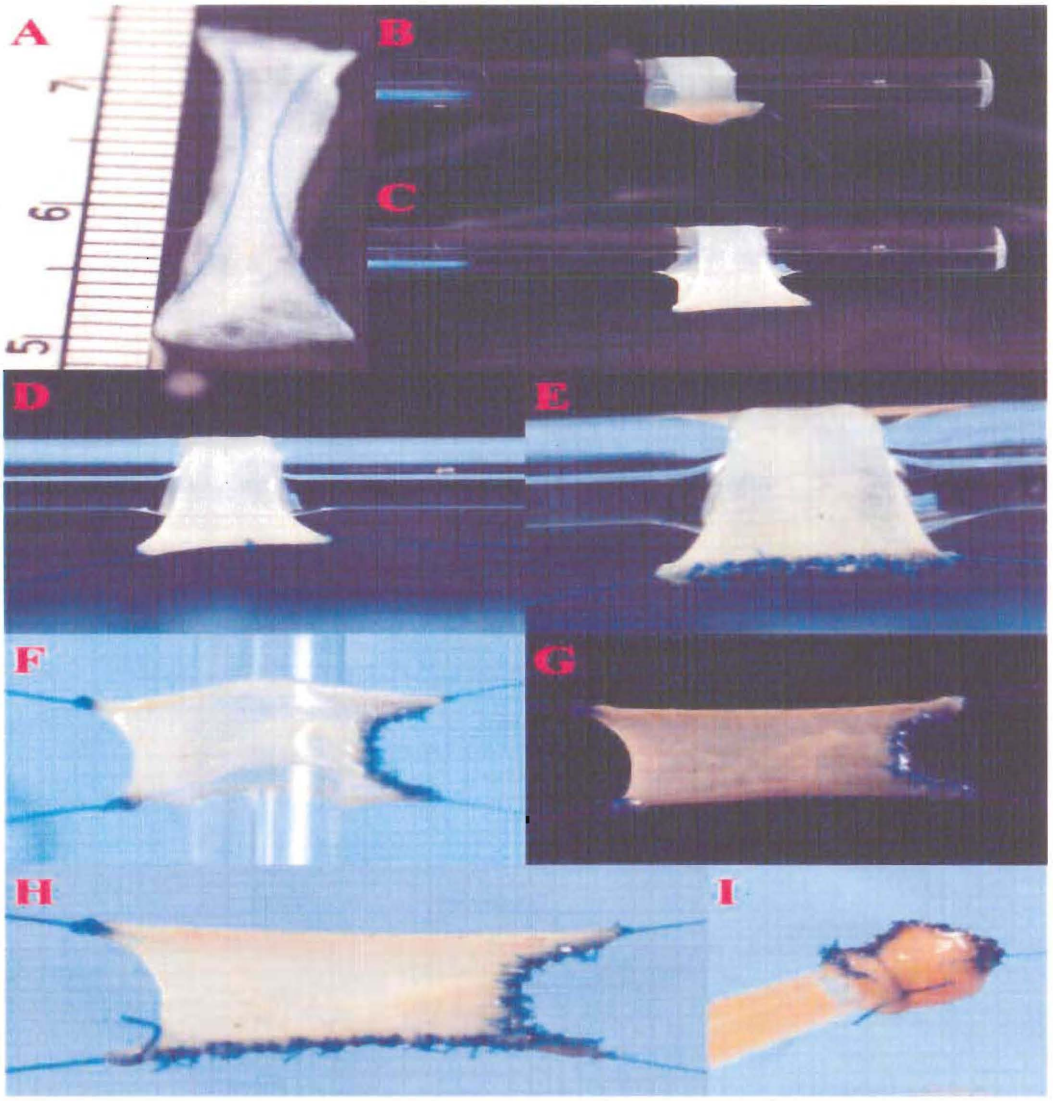

Figure 2: Preparing the SIS packet. The initial lengths of suture material have been passed through the SIS just prior to it being arranged on the glass rod (A). The SIS is now looped around a 5mm diameter glass rod (B) and secured with a knot at each corner (C). Using the lengths of suture material to manipulate the SIS the first stitch was placed (D). In similar fashion the entire length of the packet was sutured closed (E). With lengths of suture secured at each of the 4 corners (F) it is now possible to remove the SIS from the glass rod (G). Once the SIS tube was appropriately arranged another open side was

sutured closed (H). The integrity of the SIS packet was tested using an aqueous orange dye (I).

2.4. Isolation of Pancreatic Cells

The pancreases were removed from adult male Sprague Dawley rats (Charles River, Wilmington, Mass) immediately after they were euthanized in a stainless steel, gasketed, CO_2 chamber, washed in Krebs Henseleit buffer (KHB) (Sigma Chemical Company, St. Louis, MO), and then placed in a solution containing 30mg of Sigma Type V collagenase from clostridium histolyticum (Sigma Chemical Company, St. Louis, MO) and 15ml of (KHB). Each pancreas was then finely divided manually, and placed in 37°C metabolic shaker for 2.5 hours while infused with 95% O2; 5% CO_2 to disrupt the connective tissue matrix, and isolate the pancreatic parenchymal cells. If more than 3 pancreases were being digested at once, an additional 15mg of collagenase was added to the solution in the metabolic shaker and an additional hour was allowed for complete digestion. At the end of the digestion period the entire solution was filtered through a 210μm filter using surgical suction. The filtrate was placed in a 10°C centrifuge for 5 minutes at 500 rpm. After the supernatant was removed and the pellet was resuspended, a 50μL aliquot of the solution was removed and diluted with 150μL of KHB. Trypan blue, a vital stain that is excluded from viable cells was used to evaluate whether the cells were still intact prior to implantation. A manual cell count was performed using a hemocytometer to determine the approximate number of cells present in each prepared isolate. Standard dilutions of the cell suspensions were then aliquoted into each of the SIS

packets, which were then closed using a similar microsuturing technique as used initially to construct the SIS packet. A small sample of the pancreatic cell isolate was placed in formalin preservative until it could be prepared, examined and compared to the growth within the SIS packets.

2.5 SIS Packet Implantation

The recipient rats were induced and anesthetized to a surgical plane of anesthesia using ketamine (40 mg/kg, IM) and meditomidine (.4 mg/kg, IM) administered using a 22 gauge needle and syringe. A sterile field was established and aseptic procedures were used throughout the procedure. A 2 cm, longitudinal, abdominal incision was made through the linea alba to enable implantation of one SIS packet in the abdominal cavity. The length of suture and needle that were retained on the packets were used to secure them a few centimeters away from the edge of the incision (Figure 3). The incisions were closed with interrupted sutures using 2.0 silk on a cutting needle for the muscular layer and interrupted subcuticular sutures using 4.0 Vicryl on a cutting needle for the skin closure. A second SIS packet was implanted in the scrotum of each recipient animal through a 2cm longitudinal incision. This incision was closed with 5.0 Vicryl on a cutting needle using a continuous subcuticular suture. An initial dose of butorphanol (2 mg/kg, SQ) was administered 30 minutes prior to emergence from anesthesia to control pain. The animals were monitored until dorsally recumbent after which they were returned to the Laboratory Animal Resource Center (LAR) and placed on normal food and watering schedules. A second dose of Butorphanol (2 mg/kg, SQ) was administered 4 hours after

the initial dose for continued pain control. During the first 24 hours post surgery the recipient rats were observed every 8 hours, looking for any signs of pain or discomfort. After the first 24 hours the recipient rats were monitored daily for the next 4 days, once again looking for signs of pain or discomfort, after which they were monitored by the animal colony staff.

Figure 3: Implantation of the SIS packet. (A) The green arrow points to an SIS packet that has been filled with pancreatic cells just prior to implantation. (B) The green arrow now points to the SIS packet as it is being secured in the abdomen.

2.6. Explantation Procedure

The recipient rats were euthanized in a stainless steel, gasketed, CO_2 chamber prior to removal of the SIS packet. Death was confirmed by opening the chest cavity and observing cardiac stasis. Incisions were made near the implantation site to allow access to the abdominal cavity and scrotum. Through these incisions the SIS packets were located and removed. If a large amount of excess tissue was present it was carefully removed to ensure the SIS packets and their contents were not disturbed. The tissue samples were

then placed in formalin prior to standard dehydration, infiltration and paraffin embedment.

2.7. Tissue Processing and Embedding of Tissue Samples

A modified technique was employed to process the isolated pancreatic cells saved in formalin. In order to prevent these cells from escaping through the slits of the embedding cassettes they were placed in a lens paper envelope prior to processing. The paraffin blocks were sectioned at a thickness of 5μm prior to staining.

2.8. Staining Procedure

Maldonado's Method for the staining of pancreatic cells was used during this experiment due to its staining specificity for the various pancreatic cell types; alpha cells stain purple, beta cells stain blue, delta cells stain light blue with granules and exocrine cells stain grayish blue with red granules (Eng Scientific, Inc., Clifton, NJ). Such a specific stain was used because the digested pancreatic cells were not organized as they would be in an intact pancreas making identification of specific pancreatic cells amongst SIS and other tissue components very difficult. The use of this stain was intended to assist in identifying pancreatic tissue present at the time of explantation.

2.9. Examination Procedure

The tissue sections were then examined using light microscopy under 10X, 20X, 40X and 60X magnification.

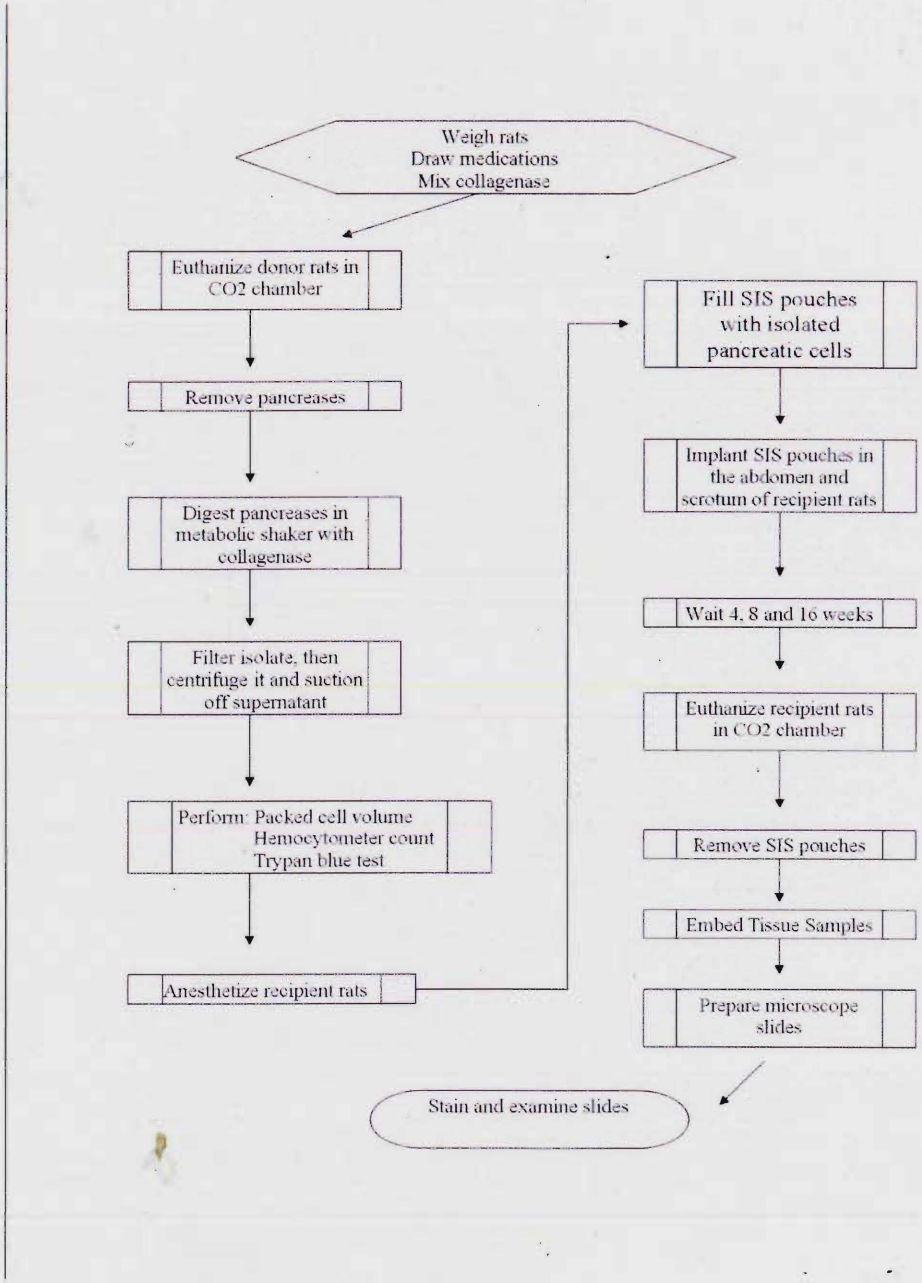

Figure 4: Flow chart of experimental procedure.

2.10. General Statistical Approach

Inferential statistics were used to evaluate the proportion of SIS packet found to contain viable cells following 8, 12 and 16 weeks post SIS packet implantation. Since each animal will receive 2 SIS packet, this study will consider the implantation site as the sampling unit in assessing response. The lower limit of the 95% binomial confidence interval was used in evaluating the a priori level of response. If the lower boundary exceeds the theoretical level, there is a <5% that the observed response is equivalent to the a priori threshold.

2.11. General Rules for Data Handling of Missing, Unused or Inconsistent Data

No method of data imputation will be ascribed for missing of inconsistent data.

2.12. Handling of Dropouts or Missing Data

All animals will be included in the primary reconciliation summaries. No method of imputation will be used for missing data. All available data from animals that fail to complete will be included in the overall data summaries.

2.13. Data Transformation Before Analysis

No data transformations are planned or anticipated; all data will be taken at face value and reported accordingly.

2.14. Stopping Rules for the Study

Estimates were prepared based on the proportion of responders within the single treatment arm against an a priori level of response. In order for the graft materials and associated surgical procedure to be considered feasible for continued research and development, minimum success rates from 1% to 10% were considered. With a power of 80% and an alpha of 5% minimum of 12 implantations per site would be needed to detect a difference in response of 15% to 39%. Animals in which there is a post surgical wound dehiscence will be euthanized by CO_2 inhalation and included in the "non-success" tally.

RESULTS

3.1. Trypan Blue Test

The isolated cells excluded Trypan Blue indicating the cell membranes were still intact. While under microscopic examination it was noted that α and β cells, as well as pancreatic acini, were present as individual cells and in groups of a few cells.

3.2. Hemocytometer Count

On 3/7/08 the pancreatic cell isolate contained 14,700,000 cells/mL, this was the most concentrated isolate obtained. In contrast, the least concentrated isolate contained 5,500,000 cells/mL and was obtained on 2/21/08 (Table 1).

Table 1: Concentrations of pancreatic cell isolate.

Date Isolated	Implant Number(s)	Cells Counted	Cells/mL
2/14	4-1	93	13 950 000
2/21	4-2; 4-3; 4-4	37	5 550 000
2/29	3-1; 3-2; 3-3	70	10 500 000
3/7	3-4; 2-1; 2-2	98	14 700 000
3/11	2-3; 2-4	85	12 750 000

3.3. Maldonado's Stain

Maldonado's staining method was originally chosen because of its ability to differentially stain the various cell types of the pancreas (Eng Scientific, Inc., Clifton,

NJ). However, since the methodology employed in this experiment was not successful in supporting the growth of pancreatic cells, Maldonado's staining method was not employed as originally intended. Rather, Maldonado's staining method was unexpectedly successful in differentially staining connective tissue, skeletal muscle, adipose tissue and sebaceous glands. Therefore, it still proved beneficial by differentiating tissue types beyond its traditional application in the pancreas.

3.4. Histologic Comparison of Growth by Implantation Site

Examination of tissue samples taken from the implantation sites revealed that cellular proliferation did occur. At the margins of the SIS packet, varying degrees of tissue growth and SIS remodeling was noted. Blood vessels were present in this new tissue indicating that a degree of neovascularization had occurred. However, it quickly became apparent that the type of tissue observed varied by implantation site. It was quickly determined that the abdominal implants demonstrated abundant skeletal muscle growth, as evidenced by the classic striated pattern of that tissue type, and a relative paucity of glandular tissue. In comparison, the scrotal implants displayed clusters of glandular looking cells. These clusters of cells were present in varying degrees of frequency and size, but the appearance of the cells remained constant throughout. The scrotal implants were therefore more likely to have provided the proper environment for pancreatic cell proliferation but we were unable to definitively prove that the cells observed were pancreatic in origin from these initial observations (Figure 5). Further

histologic comparison of the cells in the scrotal implants was necessary to more accurately determine their origin (Table 2).

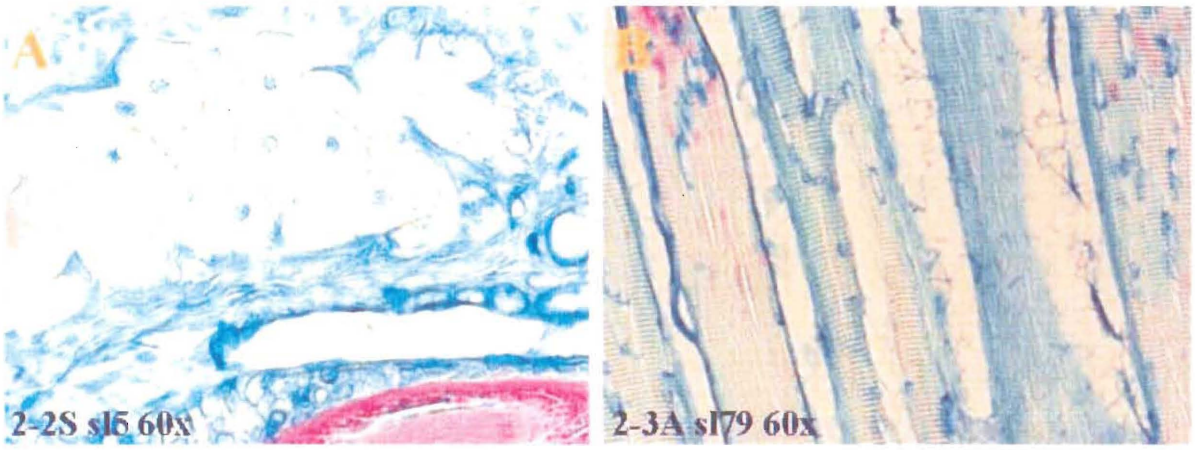

Figure 5: Scrotal implants (A) exhibited more of a glandular growth pattern, whereas abdominal implants (B) displayed skeletal muscle growth.

Table 2: Comparison of growth by implantation site. Much more possible pancreatic cell growth was noted in the scrotal implants than in the abdomen implants. However, further examination of the tissue sample from the scrotum was necessary to determine its origin.

Scrotal Implantation	
Sample Number	Result
2-1	+
2-2	+
2-3	+
2-4	+
3-1	-
3-2	+
3-3	+
3-4	+
4-1	+
4-2	N/A
4-3	+
4-4	-

Abdominal Implantation	
Sample Number	Result
2-1	-
2-2	-
2-3	-
2-4	-
3-1	-
3-2	-
3-3	-
3-4	-
4-1	-
4-2	-
4-3	-
4-4	-

Key: + indicates possible pancreatic cell growth

3.5. Histologic Comparison of Scrotal SIS Packet to Intact Rat Pancreas

Direct comparison of the glandular elements of the scrotal SIS packets to intact rat pancreas, which was also stained with Maldonado's method, revealed markedly different staining patterns. The intact rat pancreas stained in such a way as to reveal a light purple background with islands of more darkly stained tissue throughout. Even on 60X magnification it was difficult to identify clear demarcations between the acini. In comparison, the glandular cells of the scrotal implants stained light blue and the acini were clearly visible, as were granules within the acini. There was also a more darkly staining centrally located structure in the cells from the scrotal implant that was not present in the pancreatic tissue. When comparing the size of the structures observed in both tissues it was readily observable that the cells isolated in the scrotal implant were much larger than those in the pancreas. Based on these morphologic characteristics we do not believe that the cells within the scrotal implant are pancreatic in origin (Figure 6).

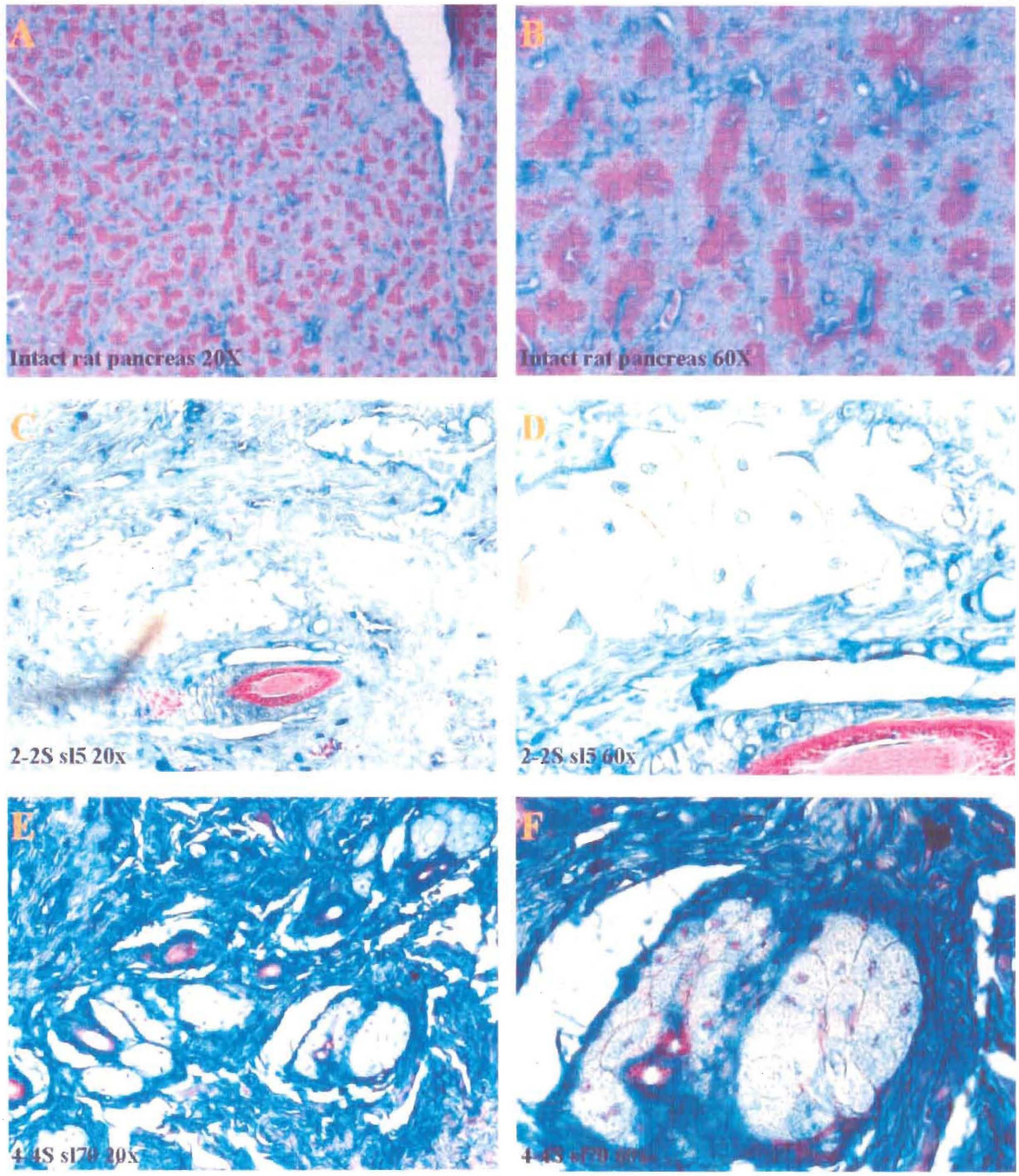

Figure 6: Morphological comparisons indicate that cells from the scrotal SIS implants (C, D, E & F) are not pancreatic in origin (A & B).

3.6. Histologic Comparison of Scrotal SIS Packet to Rat Skin

The cells identified in the SIS packets implanted in the scrotum were next compared to rat sebaceous glands which revealed that the cells were morphologically similar. The size of the acini as well as the staining characteristics of the acini is similar in both tissue samples. In both cases the acini have a more lightly staining periphery while the center stains more darkly. Granules are also visible in the acini from both locations further supporting the theory that the scrotal implanted SIS packets induced the proliferation of recipient sebaceous glands (Figure 7).

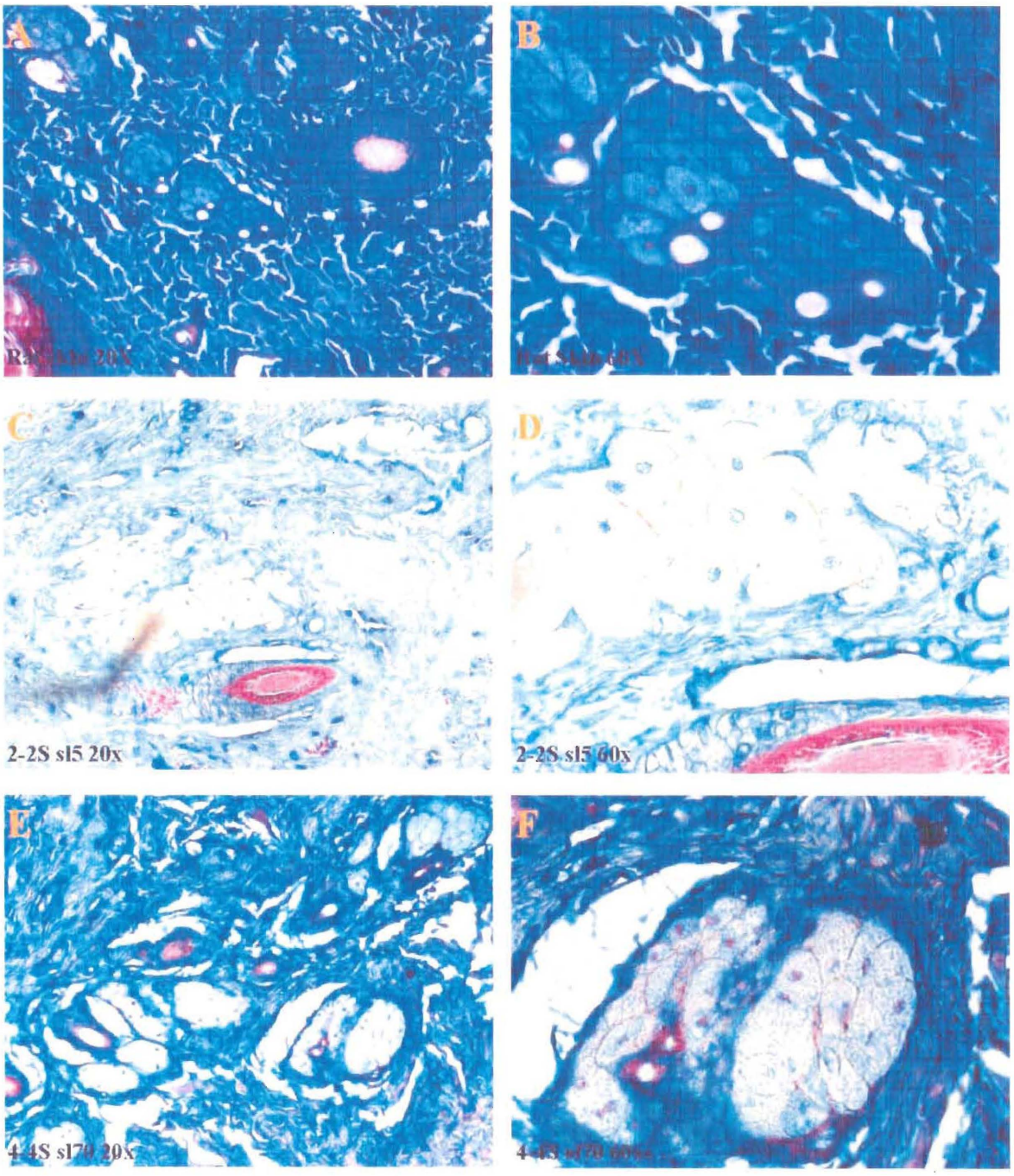

Figure 7: Sebaceous glands (A & B) resemble the cells identified in the scrotal SIS packets (C, D, E & F)

3.7. Statistical Analysis

The above stated results precluded any mode of quantative or qualitative statistical analysis aimed at determining which implantation site was more successful in promoting the growth of pancreatic cells in vivo. However, it is possible to examine the results in a different light than originally anticipated. We can now compare the amount of native tissue proliferation observed at each of the two implantation sites with respect to the duration of implantation and the relative success of porcine SIS to serve as suitable bioscaffold for recipient abdominal skeletal muscle and scrotal tissue.

3.8. Consideration of Growth at Month Two

When examined two months post implantation all of the SIS packets were supporting cell growth and proliferation. Abdominal SIS packets were being remodeled into skeletal muscle as evidenced by the striations present in some samples. Scrotal SIS packets allowed sebaceous glands present in the recipient scrotal tissue to proliferate (Figure 8). Therefore, 2 months post-implantation in either the abdomen or scrotum SIS was supportive of native cell growth (Table 3).

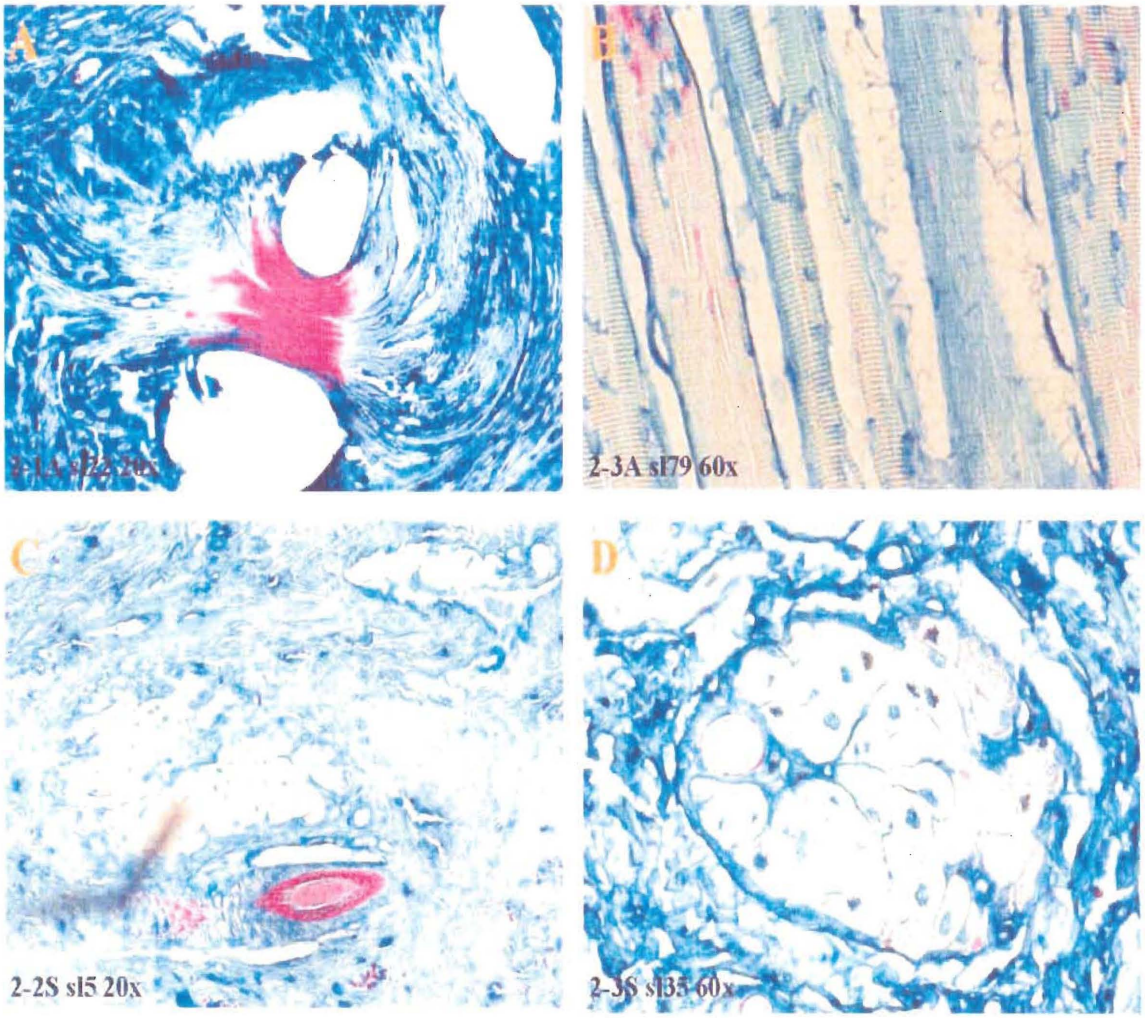

Figure 8: At 2 months clear evidence of skeletal muscle in abdominal implants (A & B) and sebaceous glands in scrotal implants (C & D) was present.

Table 3: Two months post-surgery 100% of scrotal and abdominal implants supported growth of native tissue.

2 Month Interval	
Sample	**Result**
2-1A	Skeletal Muscle
2-2A	Skeletal Muscle
2-3A	Skeletal Muscle
2-4A	Skeletal Muscle
2-1S	Sebaceous Gland
2-2S	Sebaceous Gland
2-3S	Sebaceous Gland
2-4S	Sebaceous Gland

3.9. Consideration of Growth at Month Three

The abdominal implants once again exhibited extensive skeletal muscle growth in 100% of samples. In fact, it appeared that skeletal muscle was beginning to replace the SIS. Three of the four (75%) scrotal implants possessed cells identical to those present in the 2 month sample group (Table 4). It is important to note that these cells not only became more numerous in terms of the number of cells in individual clusters but also in the number of clusters present (Figure 9).

Table 4: Abdominal and scrotal implants still supported native tissue growth 3 months post-implantation.

3 Month Interval	
Sample	**Result**
3-1A	Skeletal Muscle
3-2A	Skeletal Muscle
3-3A	Skeletal Muscle
3-4A	Skeletal Muscle
3-1S	-
3-2S	Sebaceous Gland
3-3S	Sebaceous Gland
3-4S	Sebaceous Gland

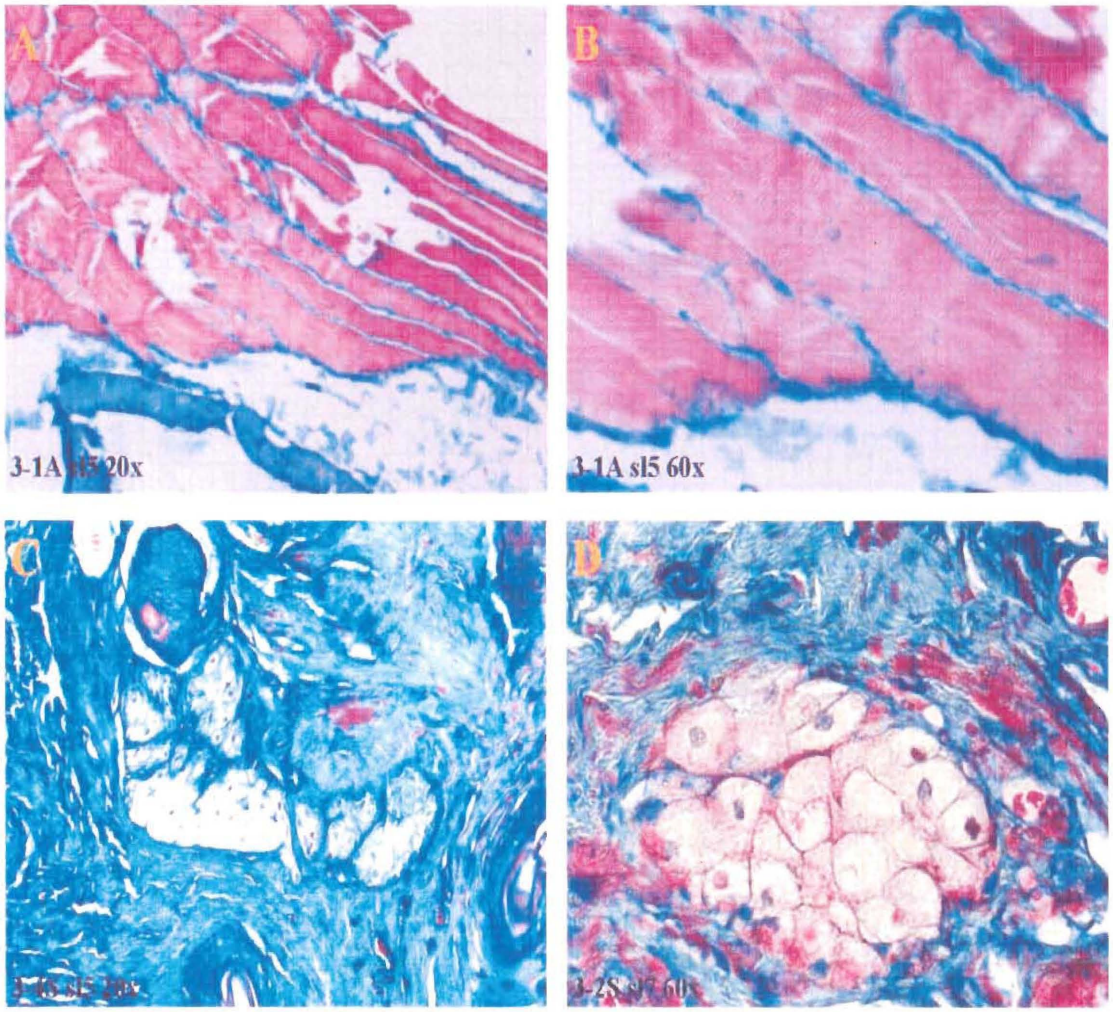

Figure 9: Three months post surgery even more skeletal muscle (A & B) and sebaceous gland (C & D) growth was observed.

3.10. Consideration of Growth at Month Four

A similar trend was observed 4 months post-implantation in that native tissue growth was supported. As previously, 100% of abdominal implants supported skeletal muscle growth. The scrotal implants still supported sebaceous gland growth but to state this figure as a percentage would produce a deceptively low number as 1 of the 4 tissue samples was not available for examination (Table 5). Once again the number of cells as well as the number of cell clusters increased dramatically over the previous time interval. In fact, when examining the 4 month samples it was sometimes necessary to use the 10X lens in order to visualize the degree of growth without making it artificially low by excluding some growth that was only visible on the periphery of the field when using the 20X lens (Figure 10).

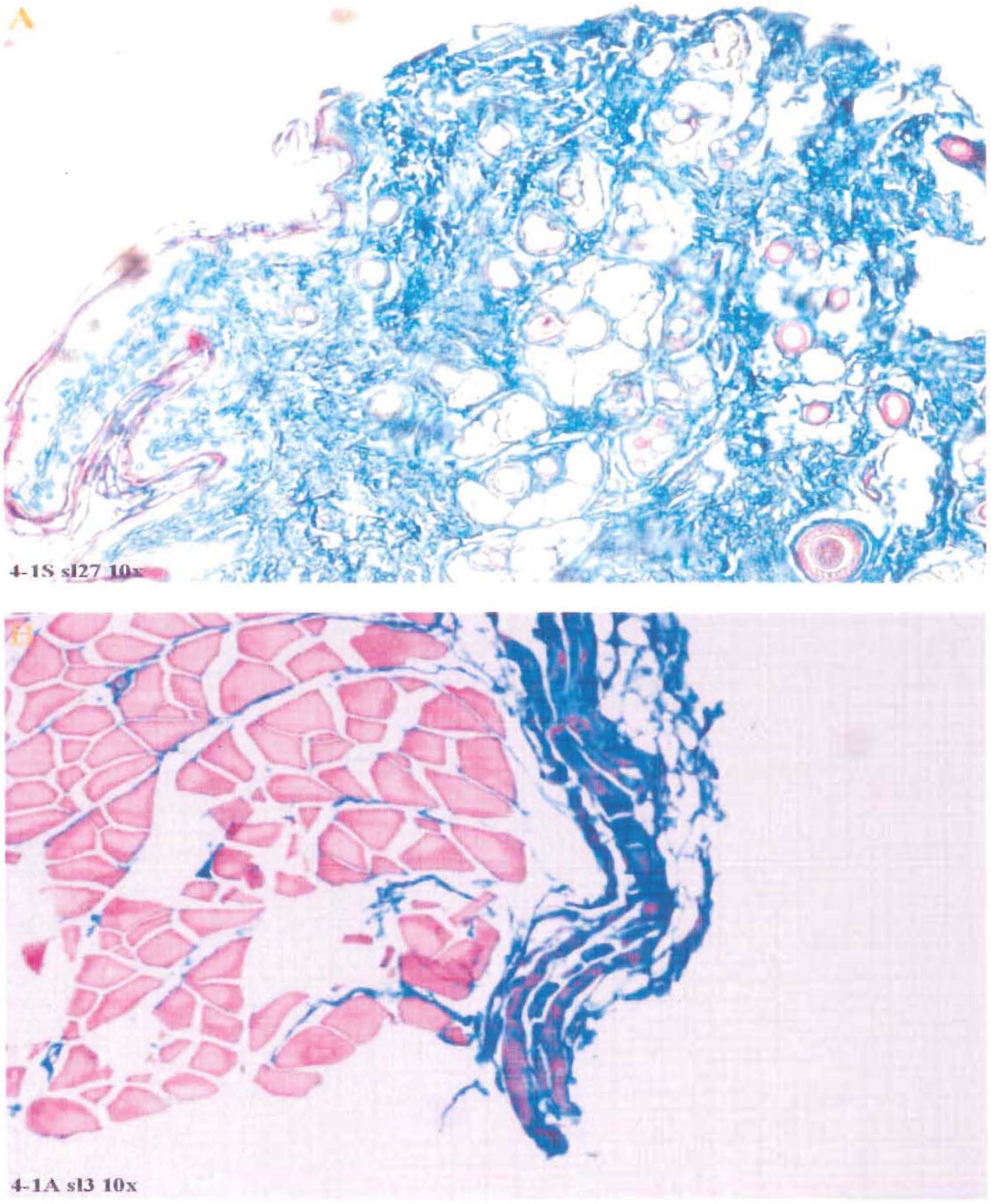

Figure 10: The trend continued at 4 months, wherein even larger quantities of skeletal muscle and sebaceous glands were observed. In fact, lower magnification had to be used to accurately depict the degree of sebaceous gland and skeletal muscle proliferation.

Table 5: Once again native tissue growth was supported in nearly 100% of examined implantation sites.

4 Month Interval	
Sample	**Result**
4-1A	Skeletal Muscle
4-2A	Skeletal Muscle
4-3A	Skeletal Muscle
4-4A	Skeletal Muscle
4-1S	Sebaceous Gland
4-2S	N/A
4-3S	Sebaceous Gland
4-4S	Sebaceous Gland

DISCUSSION

The methodology employed in this experiment failed to prove whether SIS is able to support and induce proliferation of transplanted pancreatic cells over an extended time frame. Even though the tissue samples at each of the three post-implantation endpoints of this study revealed no pancreatic cells, it is felt that a number of factors could have contributed to these results including the inadequate diffusion of nutrients and oxygen at the implantation site, a buildup of toxic metabolites within the SIS packet, and a lack of adequate neovascularization over time. The possibility of inadequate diffusion across the SIS membrane, which would have contributed to the buildup of toxic metabolites, is particularly likely given the fact that inadequate diffusion ratios are a limiting factor in the development of the bioartificial pancreas.

Since we observed that the native tissues proliferated, this indicates that the SIS maintained its ability to act as a bioscaffold in the implantation environment and the failure to observe the presence of pancreatic cells was not an overt failure of the SIS. Given the above observation it is possible that some of the same factors that may have prevented pancreatic cell proliferation such as temperature, pH and hormonal influences, could have aided in native tissue proliferation. Those factors, although different from those experienced in their normal anatomic location for the transplanted pancreatic cells, would have been almost identical to the conditions in which the native tissues normally exist. The addition of the SIS and associated growth factors would then have provided an environment ideally suited to allow those native tissues to proliferate. Given the propensity of SIS to promote the proliferation of tissue at the point of disruption of the

recipient tissue, it is not surprising that disrupted native tissue used the SIS as a bioscaffold for its own proliferation.

In order to more completely explain how the proliferation of native tissues occurred we must consider the circumstances under which the native cells could have been disrupted and introduced to the SIS. The most likely scenario in which this could have occurred in the abdomen was when tightly securing the SIS packet to the abdominal wall, as it was placed in intimate contact with the disrupted abdominal musculature at the needle puncture site. Other possibilities would be that native tissue cells could have been transferred from surgical instruments or gloves to the packet during the implantation procedure or direct contact of the SIS packet with disrupted native cells could have occurred when the SIS packet touched the incision line at either site. This direct contact scenario is particularly likely with respect to the scrotal implants based on the fact that the incision was not as large or as linear in the scrotum as compared to the abdomen. This made it technically more difficult to place the packet without it coming into contact with the native scrotal sebaceous glands. Another possibility is that after the SIS packets had been implanted in their respective locations the suture material used to construct the packet from the SIS sheet could have caused disruption of the tissue immediately surrounding the implantation site through frictional rubbing thereby providing a larger quantity of disrupted cells access to the SIS over an extended period of time.

Once the native tissue cells contaminated the SIS packet they were placed in an environment nearly identical in terms of temperature, pH, and hormones to their natural environment. Since SIS has been shown in numerous tissues to act as a bioscaffold by supporting the growth of any disrupted tissue with which it comes into contact, it is not

surprising that the native cells remained viable. In fact, the growth of the native cells could have been enhanced by (1) the presence of SIS; (2) growth factors associated with the SIS; (3) the functioning of SIS in vivo as a filtering membrane; and (4) the orientation of the SIS with its mucosal surface facing outward and functioning as a one way-valve for the absorption of nutrients (Figure 10 & 11). With the mucosal-facing side oriented to the outside of the packet it is conceivable that the penetration of the native cells to the interior of the SIS packet was enhanced, placing even more cells into a highly modified environment. Once the native cells were introduced to the SIS and began to proliferate, they would have further altered the implantation environment by consuming available nutrients and growth factors, introducing toxins and further altering the pH. These factors combined with the ever increasing number of native cells could have easily overwhelmed the pancreatic cells that were implanted. It is therefore conceivable that if the implanted pancreatic cells initially began to proliferate, the more quickly proliferating native cells further altered the environment in such a manner as to inhibit pancreatic cell proliferation. This further altered environment would have encouraged the proliferation of native cells allowing the native cells to overcome and eventually replace the transplanted cells as the dominant cell type as seen in our tissue samples.

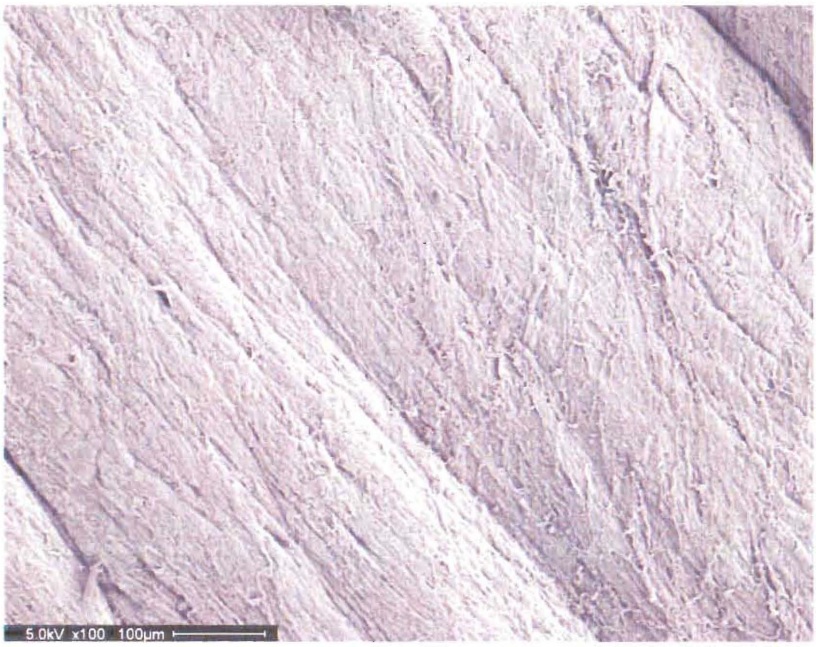

Figure 11: Scanning electron micrograph 100X - mucosal surface. Courtesy of Charlotte Greene PhD

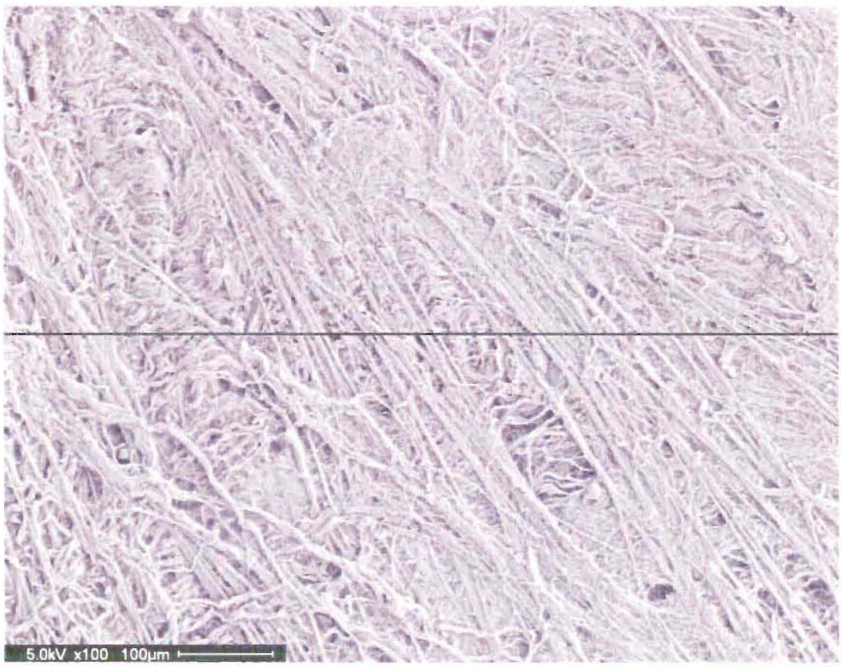

Figure 12: Scanning electron micrograph 100X - serosal side. Courtesy of Charlotte Greene Phd

The methods employed in the experiment and the subsequent results have raised a number of questions that warrant further consideration prior to the conduction of any follow up experiment. Some of those questions include; was the inability of the pancreatic cells to proliferate due to a failure of the SIS to provide an adequate bioscaffold for the proliferation of pancreatic cells? Was the lack of pancreatic cell proliferation and abundant native cell proliferation the result of the method used to surgically place the packets? Was a site distant from the pancreas' normal anatomic location not an appropriate physiologic environment to promote pancreatic cell proliferation? Were the pancreatic cells present and we just could not see them amongst all the connective tissue and native cells. One possible way to answer these lingering questions would be to conduct a similar experiment with any number of potential procedural modifications that would eliminate some of the potential pitfalls of the experimental design and allow us to more accurately determine what was occurring at various points post implantation.

In order to determine if SIS could function as a bioscaffold for pancreatic cells it would be useful to design an experiment with initial endpoints within the first few hours, days and then weeks post implantation. This would allow us to examine some of the more immediate effects implantation had on the pancreatic cells, possibly allowing us to determine if the pancreatic cells died shortly after being placed in an inhospitable environment or if their growth was simply overtaken by that of the native tissue. Since the first end point in this study was two months post-surgery we were unable to observe any of the pancreatic cells' initial response to being placed in the SIS packet. All we can definitively state is that at two months post-surgery, as with each consecutive month, no

pancreatic cells were viable; instead, there was an abundance of native tissue, namely skeletal muscle in the abdomen and sebaceous glands in the scrotum. Based on these results we can conclude that the SIS used to construct the SIS packets maintained its ability to act as a bioscaffold throughout the duration of the experiment. This finding is important because to date (Sept 2010) the ability of SIS to promote the proliferation of skeletal muscle has been demonstrated, but such activity with sebaceous glands has not been noted. More directly related to this study, it is possible to conclude that the failure of the pancreatic cells to proliferate was not a failure of the SIS to maintain its ability to act as a bioscaffold, but a failure of some other factor.

Another potential area of refinement would be the surgical techniques used in order to minimize contamination of the SIS packet with native cells. This refinement could begin by constructing the SIS packet with the assistance of Dermabond or other such surgical adhesives to minimize the amount of tissue disruption that the SIS packet would cause after it had been implanted. Surgical instrumentation could also be used to hold the incision sites open reducing the possibility that the SIS packet would contact the incision line during implantation. It would also be prudent to consider placing the SIS packets within close proximity to the pancreas, thereby providing it with an environment that more closely resembles it normal physiologic environment. Directly suturing the SIS packet to the pancreas would take advantage of the fact that SIS is known to promote the proliferation of disrupted tissues, the disruption being caused by the suture material puncturing the pancreas.

It would also be worth considering different options for locating the pancreatic cells upon removal of the SIS packet, as well as determining their functionality in vivo.

Rather than preparing the tissue samples for histologic examination as done in this experiment, it is proposed that digesting the SIS packets and their contents in the same manner as used to initially to isolate the pancreatic cells from the donor animals, may make identification of any pancreatic cells that are present in the tissue samples upon removal from the recipient animal easier. If one was to use Maldonad's staining method again it would be beneficial to successfully stain a sample of the pancreatic cell isolate to allow more ready comparison of the SIS packets contents to individual pancreatic cells rather than to intact rat pancreas. Immunohistochemical staining of the isolated cells prior to implantation would not only make identification of pancreatic cells within the tissue samples easier but might also aid in differentiating the original cells from any that arose as the result of regeneration of disrupted tissues. Monitoring the blood levels of the various pancreatic secretions may enable us to monitor the functionality of the implanted cells. It may also be prudent to consider the use of a different animal model, potentially hyperinsulinemic rats whose pancreatic cells have already shown a propensity to proliferate.

Given all the questions brought about by the results of this study is impossible to determine with absolute certainty the effects of SIS on the long term viability of transplanted pancreatic cells. Further experiments that take into account some of the lessons learned while conducting this experiment may be better able to provide definitive answers as to the ability of SIS to support pancreatic cells.

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